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Intracellular Oceanospirillales inhabit the gills of the hydrothermal vent snail *Alviniconcha* with chemosynthetic, γ-Proteobacterial symbionts

R. A. Beinart,[1] S. V. Nyholm,[2] N. Dubilier[3] and P. R. Girguis[1]*

[1]*Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA.*
[2]*Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA.*
[3]*Symbiosis Group, Max Planck Institute for Marine Microbiology, Bremen 28359, Germany.*

Summary

Associations between bacteria from the γ-Proteobacterial order Oceanospirillales and marine invertebrates are quite common. Members of the Oceanospirillales exhibit a diversity of interactions with their various hosts, ranging from the catabolism of complex compounds that benefit host growth to attacking and bursting host nuclei. Here, we describe the association between a novel Oceanospirillales phylotype and the hydrothermal vent snail *Alviniconcha*. *Alviniconcha* typically harbour chemoautotrophic γ- or ε-Proteobacterial symbionts inside their gill cells. Via fluorescence *in situ* hybridization and transmission electron microscopy, we observed an Oceanospirillales phylotype (named AOP for '*Alviniconcha* Oceanospirillales phylotype') in membrane-bound vacuoles that were separate from the known γ- or ε-Proteobacterial symbionts. Using quantitative polymerase chain reaction, we surveyed 181 *Alviniconcha* hosting γ-Proteobacterial symbionts and 102 hosting ε-Proteobacterial symbionts, and found that the population size of AOP was always minor relative to the canonical symbionts (median 0.53% of the total quantified 16S rRNA genes). Additionally, we detected AOP more frequently in *Alviniconcha* hosting γ-Proteobacterial symbionts than in those hosting ε-Proteobacterial symbionts (96% and 5% of individuals respectively). The high incidence of AOP in γ-Proteobacteria hosting *Alviniconcha* implies that it could play a significant ecological role either as a host parasite or as an additional symbiont with unknown physiological capacities.

Received 18 July, 2013; accepted 30 May, 2014. *For correspondence. E-mail pgirguis@oeb.harvard.edu; Tel. (+1) 617 496 8328; Fax (+1) 617 495 8848.

Introduction

In recent years, lineages from the γ-Proteobacterial order Oceanospirillales have emerged as widespread associates of marine invertebrates. In shallow-water habitats, Oceanospirillales are common and even dominant members of the tissue and mucus-associated microbiota of temperate and tropical corals (Sunagawa *et al.*, 2010; Bayer *et al.*, 2013a,b; Bourne *et al.*, 2013; Chen *et al.*, 2013; La Rivière *et al.*, 2013) and sponges (Kennedy *et al.*, 2008; Sunagawa *et al.*, 2010; Flemer *et al.*, 2011; Bayer *et al.*, 2013a,b; Bourne *et al.*, 2013; Chen *et al.*, 2013; La Rivière *et al.*, 2013; Nishijima *et al.*, 2013), and they have been detected in the gills of commercially important shellfish (Costa *et al.*, 2012), as well as invasive oysters (Zurel *et al.*, 2011). In deep-water habitats, Oceanospirillales have been found in association with hydrothermal vent and hydrocarbon seep bivalves (Zielinski *et al.*, 2009; Jensen *et al.*, 2010), polychaete worms, and gastropods from whale carcasses (Goffredi *et al.*, 2005; Johnson *et al.*, 2010; Verna *et al.*, 2010). In almost all cases, the nature of these animal-bacterial relationships remains undetermined. All cultivated members of the Oceanospirillales are heterotrophs known for their abilities to degrade complex organic compounds (Garrity *et al.*, 2005). Thus, hypotheses about the function of animal-associated Oceanospirillales have ranged from parasitic consumers of host tissue to beneficial symbionts that assist in the metabolism or cycling of organic compounds.

Here, we report a novel Oceanospirillales phylotype discovered in a survey of the bacterial communities associated with gill tissues of the hydrothermal vent snail *Alviniconcha*. *Alviniconcha* are dominant members of the animal communities at hydrothermal vents in the southwestern Pacific and Indian Ocean (Desbruyeres *et al.*, 1994; Van Dover *et al.*, 2001; Ramirez-Llodra *et al.*, 2007; Podowski *et al.*, 2009; 2010). This symbiotic host genus comprised at least five lineages (likely species) that are supported by the productivity of chemoautotrophic

bacterial symbionts, which use the reductants in vent fluids for the energy to fix inorganic carbon (Suzuki *et al*., 2005; 2006; Henry *et al*., 2008; Sanders *et al*., 2013). Dense populations of the bacterial symbionts reside intracellularly in *Alviniconcha* gill tissue and provide the bulk of host nutrition (Suzuki *et al*., 2005). *Alviniconcha* snails are typically dominated by either a γ- or ε-Proteobacterial phylotype according to their species, although some individuals from one of these species, currently called host type III, harbour relatively equal populations of two distinct γ-Proteobacterial phylotypes (Beinart *et al*., 2012). Here, via molecular surveys and microscopic examination, we identified a novel *Alviniconcha*-associated Oceanospirillales phylotype and localized it inside the gill cells of *Alviniconcha*. Additionally, we quantified its frequency and abundance in populations of three *Alviniconcha* host types (I–III), relative to the canonical symbiont phylotypes that associate with these hosts (γ-1, γ-Lau and an ε-Proteobacteria), from vents at the Eastern Lau Spreading Center (ELSC).

Results and discussion

Identification and phylogeny of an Oceanospirillales phylotype in Alviniconcha

Alviniconcha specimens were obtained from four Lau Basin hydrothermal vent fields, which are separated by 10 s of kilometres along the approximately 300 km north-south ELSC. The bacterial communities associated with the gills of ELSC *Alviniconcha* were surveyed by amplifying and sequencing 20 16S rRNA gene clones from libraries generated from the pooled tissue DNA of 30 individuals recovered from two vent fields (see Supporting Information Appendix S1). While sequences with affiliation to previously known *Alviniconcha* ε- and γ-Proteobacterial symbiont phylotypes dominated the survey (Beinart *et al*., 2012), two identical clones represented a novel phylotype from the γ-Proteobacterial order Oceanospirillales (hereafter referred to as 'AOP' for '*Alviniconcha* Oceanospirillales phylotype'). Following discovery of AOP in the clone library, we used BLASTN (Altschul *et al*., 1990) to search for AOP sequences in 16S rRNA gene pyrosequencing libraries (GenBank SRA:SRX450370, SRX450912, SRX450913, SRX450915) previously obtained from four *Alviniconcha* individuals (Sanders *et al*., 2013). This revealed one matching operational taxonomic unit (OTU) that comprised 72 sequence reads with ≥ 97% identity to our clones as well as to each other. Since only one other OTU, consisting of two comparatively short sequence reads, was classified as Oceanospirillales (94% identity to the AOP sequences), it is likely that AOP represents the most dominant Oceanospirillales associating with *Alviniconcha*.

To ascertain the relationship of AOP to other Oceanospirillales (and, more broadly, the γ-Proteobacteria, including the γ-Proteobacterial symbionts of *Alviniconcha*), Bayesian inference was used to construct a phylogeny of 16S rRNA genes (see Supporting Information Appendix S1; Fig. 1), including one fully sequenced AOP clone. The AOP sequence falls within a well-supported clade of Oceanospirillales that all have, with the exception of one clone, been found in association with diverse marine invertebrates from various habitats. The phylotypes that were closest to AOP were recovered from tropical, shallow-water corals from the Caribbean (Sunagawa *et al*., 2010) and the Great Barrier Reef (Bourne and Munn, 2005). The few cultivated representatives from this clade are members of the genera *Endozoicomonas* and *Spongiobacter*, which were isolated from sea slugs (Kurahashi and Yokota, 2007), corals (Raina *et al*., 2009; Yang *et al*., 2010; Bayer *et al*., 2013a) and sponges (Flemer *et al*., 2011; Nishijima *et al*., 2013). Although there is increasing evidence that this clade of Oceanospirillales phylotypes is specific to marine invertebrates (Fig. 1), the relationships among their members and their animal hosts, as well as their location in or on host tissue, have not yet been characterized. An exception is '*Candidatus* Endonucleobacter bathymodiolii', a parasite of hydrothermal vent mussels that has been shown to infect host nuclei, multiply and eventually burst from the organelle (Zielinski *et al*., 2009). The AOP 16S rRNA gene has 95% sequence identity to a '*Ca*. E. bathymodiolii' 16S rRNA gene sequence recovered from a Gulf of Mexico cold seep mussel.

Localization of AOP in Alviniconcha *gill tissue*

To localize AOP in *Alviniconcha* gill tissue, we examined *Alviniconcha* individuals via fluorescence *in situ* hybridization (FISH) using universal bacterial and AOP-specific probes targeting 16S rRNA (see Supporting Information Appendix S1; Fig. 2). Additionally, we used transmission electron microscopy (TEM) to describe its morphology in association with *Alviniconcha* gills (see Supporting Information Appendix S1; Figs 3 and 4). Gill tissue from three animals from the vent field ABE and three animals from the vent field Tow Cam were selected for these microscopic analyses (see Supporting Information Appendix S1).

Examination of *Alviniconcha* gills using FISH confirmed the presence of AOP in the gills of individuals recovered from the ABE vent field, but not in snails recovered from the Tow Cam vent field (Fig. 2A and B). In snails with AOP, populations of the phylotype were consistently localized in vacuoles (Fig. 4), approximately 10–40 μm in diameter, which were sporadically distributed throughout the gill filaments. These vacuoles were only found within the

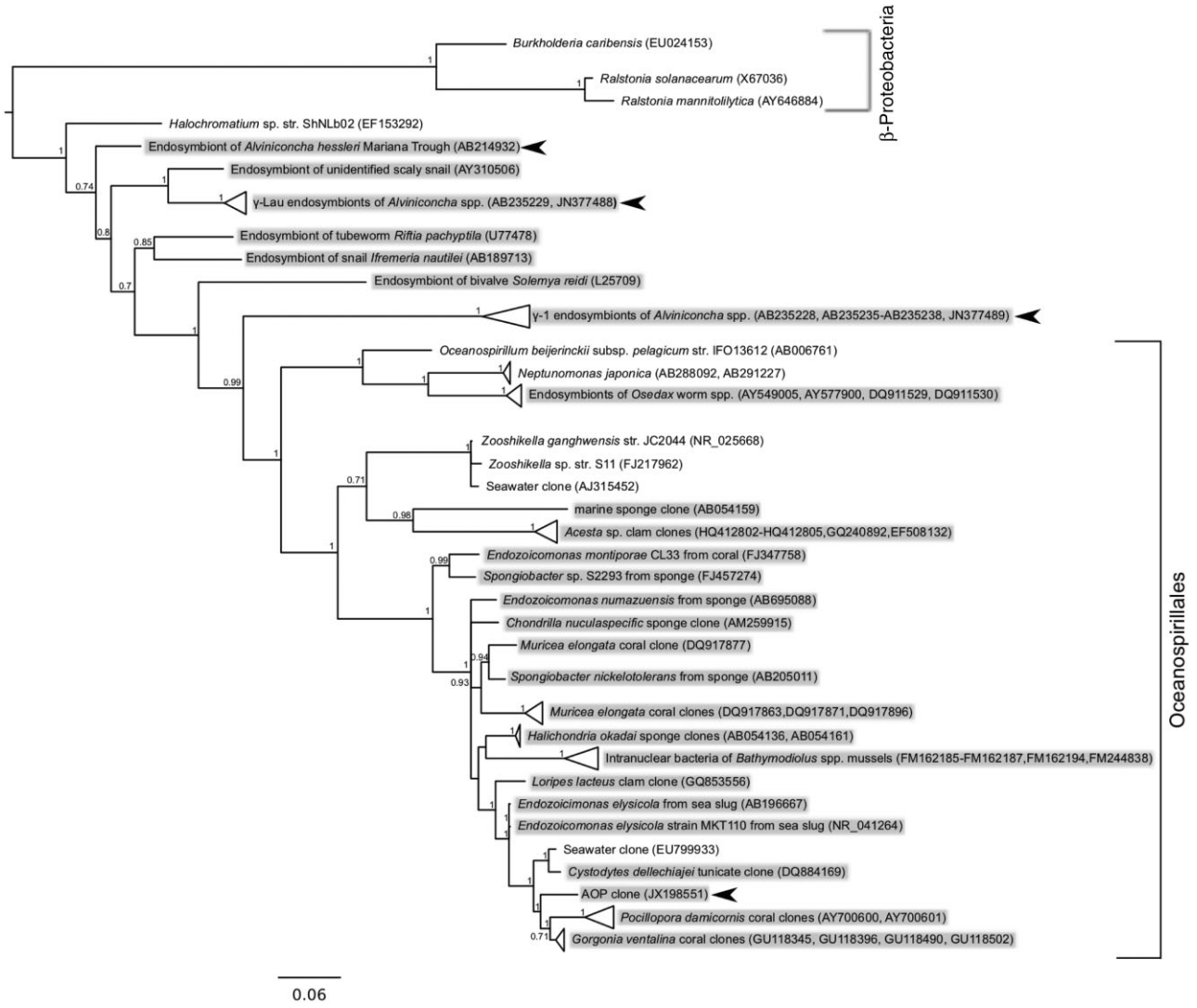

Fig. 1. A Bayesian inference phylogeny of γ-Proteobacterial 16S rRNA gene sequences showing the relationship of AOP to other Oceanospirillales, the γ-Proteobacterial chemoautotrophic symbionts of *Alviniconcha* (e.g. phylotypes γ-1 and γ-Lau) and other animals, and the sequences from the out-group β-Proteobacteria. Arrows indicate *Alviniconcha*-associated sequences. Grey highlighting indicates that the clone or strain has been found in association with a marine invertebrate. Posterior probabilities are indicated above the nodes if > 0.7.

symbiont-containing cells (bacteriocytes), typically at the apical ends of the bacteriocytes (Fig. 2C). We never observed AOP cells inside the host nuclei, which are located in the basal ends of the bacteriocytes towards the middle of the gill filaments (Fig. 2). Also, AOP was never observed in symbiont-free cells of the gill filaments, which are found where they attach to the snail's mantle or at the very ends (not shown). This contrasts sharply with the infection of the vent mussel gills by the closely related '*Ca*. E. bathymodiolii', where infection only occurs in the nuclei of symbiont-free intercalary cells that are found between bacteriocytes (Zielinski *et al*., 2009).

We also used TEM to examine the location and morphology of the bacteria inhabiting *Alviniconcha* gill tissue, revealing membrane-bound vacuoles likely containing AOP. Inspection of the gill tissue of one of the three ABE individuals also used in FISH microscopy showed that gram-negative, filamentous and rod-shaped bacterial symbionts were densely packed at the apical ends of the cells (Fig. 3A), consistent with previous descriptions of *Alviniconcha* gill morphology and the canonical symbionts therein (Stein *et al*., 1988; Endow and Ohta, 1989; Urakawa *et al*., 2005). These two morphotypes very likely represent two canonical symbiont phylotypes (although they could also reflect morphological variation within a single symbiont phylotypes), which are either free in the host cytoplasm or contained within individual vacuoles (the limitations of our preservation makes it difficult to distinguish their precise position; Fig. 3B–C). Consistent with our observations via FISH, we also found vacuoles

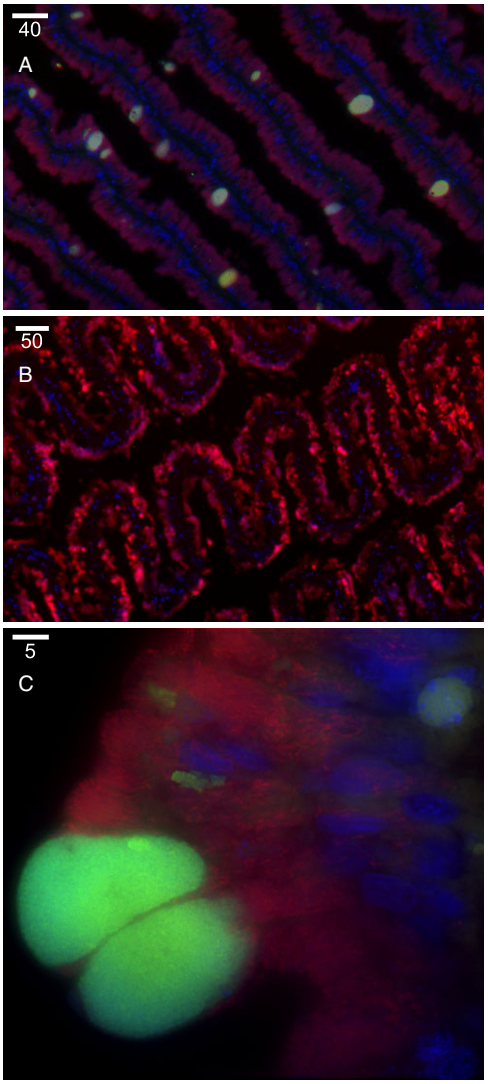

Fig. 2. Identification of AOP (green) and all bacteria, including the chemoautotrophic symbionts (red), in *Alviniconcha* gill tissue with dual FISH hybridizations using a Cy3-labelled AOP-specific probe and Cy5-labelled EUB338(I-III) respectively (Amann *et al*., 1990). The colocalization of the two probes in AOP cells results in a mixing of fluorescence signals with a yellow-green tint (Smallcombe, 2001). Additionally, all host nuclei were stained with the nucleic-acid stain DAPI and shown in blue.
A and C. Gill filaments of *Alviniconcha* from the ABE vent field with AOP-containing vacuoles.
B. Gill filament of *Alviniconcha* from the Tow Cam vent field with no AOP-containing vacuoles. All scale bars are shown in μm.

containing a third bacterial morphotype – likely the AOP phylotype – distributed sporadically throughout the gill tissue (Fig. 4). These membrane-bound compartments are full of small (~ 1 μm), irregularly coccoid, gram-negative bacterial cells that contain electron-dense particles that are somewhat similar in size and shape to those observed in '*Ca*. E. bathymodiolii' via TEM (Zielinski *et al*., 2009).

The discovery of AOP in snails from the vent field ABE, but not Tow Cam, suggested specificity for particular host lineages or their associated symbionts since there is geographic structure to the distribution of *Alviniconcha* at the ELSC vent fields. In a previous study, it was found that ABE vents are populated by an *Alviniconcha* host type that associates with γ-Proteobacterial symbionts, while the Tow Cam vents are heavily inhabited by host types that associate with ε-Proteobacterial symbionts (Beinart *et al*., 2012). Consistent with the known distribution of *Alviniconcha* host types and symbionts, we observed sulfur granules in the gills of the preserved snails from the vent field ABE (i.e. those with AOP), which to our knowledge only occurs among the γ-Proteobacterial *Alviniconcha* symbionts. Thus, our microscopic examination of AOP in gill tissue suggested specificity for the *Alviniconcha* host types with γ-Proteobacterial symbionts.

Distribution and abundance of AOP in ELSC Alviniconcha

To assess the distribution and abundance of AOP across the host types and symbiont phylotypes found at the ELSC, we used quantitative polymerase chain reaction (qPCR) to determine the abundance of AOP, relative to the canonical symbiont populations, within 283 snails, as well as their prevalence according to host type (see Supporting Information Appendix S1). As mentioned, a previous study by Beinart and colleagues (2012) demonstrated that there are three genetically distinct *Alviniconcha* host types (likely undescribed species), which form specific associations with three proteobacterial phylotypes, for a

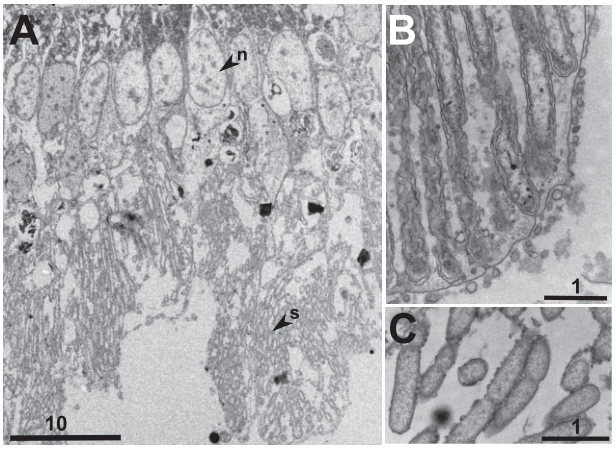

Fig. 3. Transmission electron micrographs of the symbionts of *Alviniconcha* from the ABE vent field.
A. One side of a gill filament, showing bacteriocytes, but no suspected AOP-containing vacuoles. n, host nuclei; s, symbiont cells.
B and C. Show the two symbiont morphotypes, found at the apical ends of the cells. All scale bars are shown in μm.

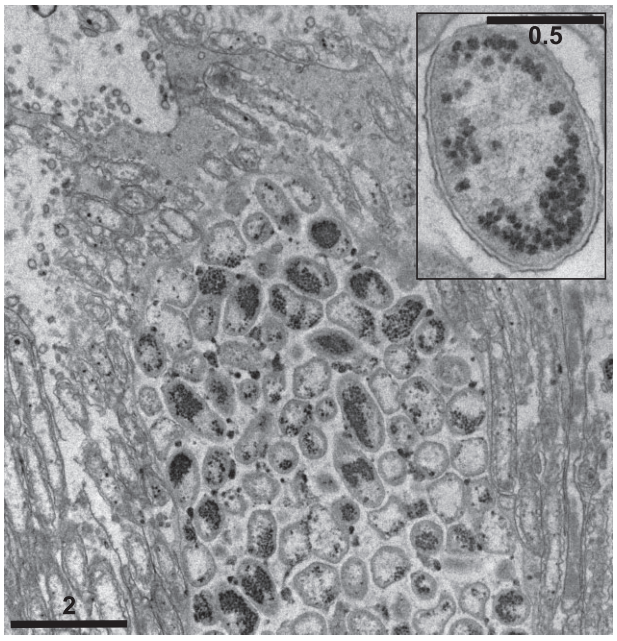

Fig. 4. A transmission electron micrograph of a suspected AOP vacuole inside a bacteriocyte of an *Alviniconcha* from the ABE vent field. Inset shows a single cell inside the suspected AOP vacuole. All scale bars are shown in μm.

total of five different host–symbiont combinations. Among the three host types, each individual snail is typically dominated by either γ- or ε-Proteobacterial symbionts, with only one of the three phylotypes representing > 99% of the detected symbiont 16S rRNA genes in a single individual. Minor, co-occurring populations of one of the other phylotypes are sometimes detected, and a small number of γ-Proteobacteria-hosting individuals associate with equal proportions of the two γ-Proteobacterial phylotypes. Across the surveyed population, AOP consistently represented a minor fraction of the total quantified 16S rRNA genes (0–36%, median 0.53%) (Supporting Information Tables S1 and S2). However, as suggested by the FISH and TEM micrographs, the prevalence of AOP differed between the *Alviniconcha* lineages dominated by ε- and γ-Proteobacterial symbionts in terms of both the frequency of occurrence and the relative abundance compared with other symbiont phylotypes. In *Alviniconcha* hosts dominated by γ-Proteobacteria (host types I and III; Beinart *et al*., 2012), AOP was detected in 96% of all screened individuals. In contrast, AOP was detected in only 5% of the *Alviniconcha* individuals hosting primarily ε-Proteobacteria (host type II, as well as a few host type I; Supporting Information Table S1). We also found a greater proportion of AOP 16S rRNA genes in *Alviniconcha* that hosted γ-Proteobacteria than those hosting ε-Proteobacteria (median 1.36% and 0% respectively; Supporting Information Tables S2 and S3). Furthermore, we observed a significant difference in the proportion of AOP 16S rRNA genes among *Alviniconcha* individuals dominated by each symbiont phylotype (Supporting Information Table S2; Kruskal–Wallis $P < 0.0001$, SPSS V20). Specifically, individuals dominated by either of the γ-Proteobacterial phylotypes (γ-1 or the γ-Lau) had significantly greater proportions of AOP than individuals dominated by the ε-Proteobacterial symbiont (Supporting Information Table S2; post-hoc Mann–Whitney U $P < 0.0001$ for both, Bonferroni-corrected $\alpha = 0.0167$, SPSS v20), while no significant difference was found between individuals dominated by the two γ-Proteobacterial phylotypes (Mann–Whitney U $P = 0.027$, Bonferroni-corrected $\alpha = 0.0167$, SPSS v20). Even within the host type (III) that can be dominated by either of the γ-Proteobacterial phylotypes, we found no significant difference in the relative abundance of AOP between individuals dominated by γ-1 or γ-Lau (Supporting Information Table S2; Mann–Whitney U $P = 0.352$, SPSS v20). Similarly, our search of previously published 16S rRNA gene pyrosequences from *Alviniconcha* hosting γ- or ε-Proteobacteria (Sanders *et al*., 2013) revealed that sequences allied to AOP were only detected in the two *Alviniconcha* that host γ-Proteobacteria (0.3% and 2% of the sequence reads). No AOP sequences, or any classified as Oceanospirillales, were detected in the two *Alviniconcha* hosting ε-Proteobacteria.

The specificity of AOP for *Alviniconcha* hosting γ-Proteobacteria was relatively consistent throughout the four ELSC vent fields (Supporting Information Table S3), despite the fact that γ- and ε-Proteobacteria hosting *Alviniconcha* are dominant at vent fields separated by 10 s to 100 s of kilometres (Supporting Information Table S3; Beinart *et al*., 2012). For example, of the 10 ε-Proteobacteria hosting individuals from ABE, a vent field that is inhabited by mostly γ-Proteobacteria hosting *Alviniconcha* with typical levels of AOP, only one had detectable AOP. Thus, even at a vent field where most of their neighbours hosted AOP, *Alviniconcha* hosting ε-Proteobacteria still had an apparent low frequency of association. This indicates that geography was not structuring the frequency of AOP in the ELSC *Alviniconcha* population, but rather that biological determinants were more important.

Overall, the observed pattern of correspondence with the γ-Proteobacterial symbionts implies that AOP interacts with these particular symbionts and/or has specificity for the two host types that associate with them. It is difficult to resolve these two options, since host and symbiont identity are linked. However, to address this issue, we compared the abundance of AOP among individuals of host type I, which can either associate with γ-Proteobacterial or ε-Proteobacterial symbionts, and found that there was no significant difference between individuals of this type that hosted the different symbiont classes (Supporting

Information Table S2; Mann–Whitney *U* $P = 0.092$, SPSS v20). This must be interpreted with caution, however, since there was a large difference in sample size between host type I individuals that hosted ε-Proteobacteria ($n = 6$) and those that hosted γ-Proteobacteria ($n = 93$), and only two of the six ε-Proteobacteria hosting individuals had detectable AOP. With that caveat, it is possible that host type may be more important than symbiont class in determining infection by the AOP.

Potential modes of interaction between AOP and Alviniconcha

Despite its low abundance, AOP has the potential to play a significant ecological role for *Alviniconcha*. Other animal-associated Oceanospirillales are known to cover the spectrum of symbiotic relationships, ranging from beneficial to harmful associations with their hosts. As an exercise in considering the modes of interaction between AOP and their hosts, here we discuss the potential role of AOP in the context of what is known about mutualistic and parasitic Oceanospirillales, although we cannot exclude the possibility that AOP has no significant effect on its host (i.e. AOP is a commensalist). In terms of parasitism, AOP is related to the intranuclear parasites of hydrothermal vent mussels (95% 16S rRNA gene identity). However, we never observed AO in host nuclei. Even if it is not nuclear-specific, it is possible that AOP represents a parasite or pathogen of *Alviniconcha* that is contained inside a membrane-bound vacuole, as is common with other intracellular pathogens (Goebel and Gross, 2001; Casadevall, 2008; Kumar and Valdivia, 2009). Alternatively, AOP may represent a minor, secondary, but mutualistic, symbiont of *Alviniconcha*. In insects, secondary symbionts are often an order of magnitude lower in abundance than the primary symbionts, but yet can confer ecologically important advantages for their hosts (Mira and Moran, 2002; Oliver *et al*., 2010). Lineages of Oceanospirillales are the intracellular mutualists of bone-eating *Osedax* worms found on whale-falls (Goffredi *et al*., 2005), as well as sap-sucking whiteflies (Thao and Baumann, 2004). In these associations, the symbionts are thought to support host nutrition through the synthesis of essential amino acids, vitamins and/or carotenoids (Santos-Garcia *et al*., 2012; Goffredi *et al*., 2014). The AOP is most closely related to *Endozoicomonas*-like phylotypes that are thought to be mutualists of healthy, tropical, shallow-water corals since they are dominant members of their microbiomes (98% 16S rRNA gene identity; Sunagawa *et al*., 2010). Recent efforts with isolates from this group of Oceanospirillales have shown that they can degrade the dimethylsulfoniopropionate (DMSP) (Raina *et al*., 2009) that is produced by the algal symbionts of corals (Van Alstyne *et al*., 2008), suggesting an important role in sulfur cycling within or around their hosts. Although DMSP production is thought to be specific to marine algae, AOP could similarly play a role in sulfur cycling in *Alviniconcha*. Thus, AOP has the potential to provide a beneficial function for the host directly (e.g. the breakdown of an organic compound consumed or produced by the host) or indirectly (e.g. by facilitating the metabolism of the other symbionts).

Conclusions

The discovery of symbioses among chemoautotrophic bacteria and invertebrates led to a watershed of research on these types of associations from many habitats, with much of the focus on the canonical, chemoautotrophic symbionts (Cavanaugh *et al*., 2006; Dubilier *et al*., 2008). Throughout ~ 35 years of research, there has been little evidence for the presence of minor microbial associates (i.e. microbes that form specific associations with their hosts but are present in low abundance, including non-chemoautotrophs). Here, through a combination of phylogenetics, microscopy and qPCR surveys, we have established that the AOP is a minor, but specific and frequent, associate of *Alviniconcha*. While the precise nature of the interaction remains to be determined, the data presented herein further extend the diversity, and potentially the functional role, of intracellular bacteria associated with *Alviniconcha*. This is the first description of an Oceanospirillales associating with *Alviniconcha* or any other hydrothermal vent gastropod and the second description of an Oceanospirillales associating with a symbiotic, hydrothermal vent mollusc. With growing awareness of the significance of microbes, either as parasites or mutualists, to organismal health and function, investigations of minor microbial associates across the known diversity of invertebrate–chemoautotrophic symbioses are warranted.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Number of *Alviniconcha* individuals in which AOP was detected/not detected via 16S rRNA gene qPCR according to their majority symbiont phylotype (ε, ε-Proteobacteria; γ-1, γ-Proteobacteria type I; γ-Lau, γ-Proteobacteria type Lau, γ-1/ γ-Lau, equal proportions of both) and host type (HT-I, -II, -III and -UD, undetermined). NA, not applicable because no individuals of this host type/symbiont combination were observed.

Table S2. Proportion of AOP 16S rRNA genes, relative to the 16S rRNA genes of the symbionts, in *Alviniconcha* individuals according to their majority symbiont phylotype (ε, ε-Proteobacteria; γ-1, γ-Proteobacteria type I; γ-Lau,

γ-Proteobacteria type Lau, γ-1/ γ-Lau, equal proportions of both) and host type (HT-I, -II, -III and -UD, undetermined). Percentages as median (minimum, maximum) are shown, followed by the number of individuals (*n*) of each host type/symbiont combination.

Table S3. Proportion of AOP 16S rRNA genes, relative to the 16S rRNA genes of the symbionts, in *Alviniconcha* individuals at the four vent fields at the ELSC according to their majority symbiont phylotype (ε, ε-Proteobacteria; γ-1, γ-Proteobacteria type I; γ-Lau, γ-Proteobacteria type Lau, γ-1/ γ-Lau, equal proportions of both) and host type (HT-I, -II, -III and -UD, undetermined). Percentages are shown as median (minimum, maximum), followed by the number of individuals in brackets. NA, not applicable because no individuals of this host type/symbiont combination were found at that vent field. The predominant host type/symbiont phylotype combination for each vent field is shaded in grey.

Appendix S1. Supplementary experimental procedures.